VIROLOGY 223, 19–28 (1996)
ARTICLE NO. 0451

Replication and Pathogenicity after Intranasal and Intracranial Inoculation of Swine with a Recombinant Pseudorabies Virus Containing a Deletion at the UL/IR Junction

H. J. DEAN,*,[1] J. M. MILLER,† M. R. ACKERMANN,‡ X. Y. GAO,§ L. L. ANDERSON,‖ C. D. JACOBSON,¶ and A. K. CHEUNG*,[2]

*Virology Swine, †Respiratory and Neurologic Disease, and ‡Avian and Swine Respiratory Diseases Research Units, National Animal Disease Center, USDA, Agricultural Research Service, Ames, Iowa 50010; §Shanghai Animal and Plant Quarantine Bureau, People's Republic of China; Departments of ‖Animal Science and ¶Anatomy, College of Veterinary Medicine, Iowa State University, Ames, Iowa, 50011

Received February 8, 1996; accepted June 12, 1996

Pseudorabies virus (PRV) is a neurotropic herpesvirus of swine. Previously, we described construction of a recombinant strain of PRV (LLT$\beta\Delta$2) which contains a 3.0-kb deletion spanning the junction of the unique long and internal repeat sequences. Compared to the parental strain, Indiana-Funkhauser, and a virus rescued for the deleted sequences (LLTβres), LLT$\beta\Delta$2 replicated efficiently at the site of inoculation, yet exhibited significantly reduced virulence when inoculated intranasally in pigs. In this report, we investigated the effect of the deletion on PRV replication and virulence after intracranial inoculation of swine, in comparison to replication and virulence after intranasal inoculation, in order to more precisely locate the defect in LLT$\beta\Delta$2. Four-day-old pigs were infected intranasally with LLT$\beta\Delta$2 or LLTβres and necropsied at various times postinfection. Compared to LLTβres-infected pigs, tissue distribution of virus, PRV antigen, and lesions of LLT$\beta\Delta$2-infected pigs were comparable in all peripheral tissues examined, including trigeminal ganglia, but were reduced in tissues from the central nervous system (CNS). LLT$\beta\Delta$2 was able to replicate in the CNS after intracranial inoculation into the cerebral cortex of 2-day-old piglets and to spread from CNS to peripheral tissues. Neurovirulence of LLT$\beta\Delta$2 was somewhat reduced, as demonstrated by delayed onset of neurological signs and death in intracranially inoculated pigs. These results indicate that decreased neurovirulence after intranasal inoculation is not due to inability of LLT$\beta\Delta$2 to replicate in CNS tissues. The difference in the amount of antigen detected in CNS tissues after intracranial inoculation compared to intranasal inoculation suggests that one defect in LLT$\beta\Delta$2 is reduced ability to spread from peripheral neurons to the CNS after intranasal inoculation.

INTRODUCTION

Pseudorabies virus (PRV) is a neurotropic virus of swine which is a member of the alphaherpesvirus family, related to bovine herpes virus type 1 (BHV-1), and the human viruses herpes simplex virus (HSV) and varicella zoster virus. During natural infection of swine, PRV is spread by aerosol or contact and enters the host animal via the upper respiratory system (reviewed in reference Wittman and Rziha, 1989). The virus replicates in epithelial cells of the nasal and oropharyngeal mucosa and is disseminated from the oronasal region to the central nervous system (CNS) primarily via retrograde transneuronal transport across synaptic junctions (Babic *et al.,* 1994; Card *et al.,* 1990; Card and Enquist, 1994). After intranasal inoculation, PRV spreads to the CNS from the nasal mucosa via the olfactory nerve and from the pharyngeal mucosa through trigeminal, sympathetic, and parasympathetic pathways (Babic *et al.,* 1994; Martin and Dolivo, 1983; McFerran and Dow, 1965). Once in the CNS, PRV replicates in neurons and can also infect astrocytes and brain macrophages (Rinaman *et al.,* 1993). PRV can produce a fatal encephalitis in neonatal pigs, while older pigs generally survive PRV infection.

Pseudorabies virus is an ideal system for studying alphaherpesvirus neurovirulence because effects of genomic alterations can be evaluated in the natural host species, swine. Other recent studies on contributions of various genomic sequences to HSV and PRV neurovirulence have generally been conducted in nonhost species (rodents) (Babic *et al.,* 1993, 1994; Card *et al.,* 1990, 1992; Martin and Dolivo, 1983) or have arrived at conclusions about neurovirulence based on evaluation of pathogenicity after inoculation at peripheral sites (Kimman *et al.,* 1992; Mulder *et al.,* 1994). This study is unique because the effect of a deletion on neurovirulence and neuroinvasiveness was measured directly, by comparison of intranasal versus intracranial inoculation into the natural host species.

Previously, we constructed a recombinant strain of PRV which is significantly reduced in virulence (Dean

No endorsements are herein implied. Brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standards of the products, and the use of the names by the USDA implies no approval of the products to the exclusion of others that may also be suitable.

[1] Current address: Mallinckrodt Veterinary, Inc., 421 E. Hawley Street, Mundelein, IL 60060.

[2] To whom correspondence and reprint requests should be addressed at Virology Swine Research Unit, USDA, ARS, National Animal Disease Center, P.O. Box 70, Ames, IA 50010. Fax: (515) 239-8458.

0042-6822/96 $18.00

and Cheung, 1995). This virus, LLT$\beta\Delta$2, contains a 3-kilobase pair (kb) deletion spanning the junction of the unique long (UL) and internal repeat (IR) sequences. Known genes disrupted by the deletion include the second exon of the large latency transcript (LLT), which is expressed during latent infection of swine, and one copy of the diploid immediate early gene IE180, which is a homolog of the HSV transactivator ICP4. Compared with the parental strain, Indiana-Funkhauser (InFh), and a rescued virus, in which the deleted sequences are restored (LLTβres), LLT$\beta\Delta$2 is significantly reduced in virulence for 4-week-old and 4-day-old pigs after intranasal inoculation (Dean and Cheung, 1995). In general, younger pigs are more susceptible to PRV than older pigs. Our data also demonstrated that LLT$\beta\Delta$2 is capable of killing 4-day-old pigs but not 4-week-old pigs after intranasal inoculation. LLT$\beta\Delta$2, InFh, and LLTβres exhibit similar growth characteristics in Madin–Darby bovine kidney (MDBK) cells and in the oronasal epithelium and trigeminal ganglia (TG) of experimentally infected pigs. The purpose of the present work was to examine more precisely the defect in LLT$\beta\Delta$2 by comparing spread and replication of LLT$\beta\Delta$2 and LLTβres to and within the CNS after intracranial and intranasal inoculation of swine.

MATERIALS AND METHODS

Viruses

Construction of LLT$\beta\Delta$2 and LLTβres has been described previously (Dean and Cheung, 1995). PRV strains InFh, LLT$\beta\Delta$2, and LLTβres were propagated in MDBK cells cultured in F15 medium supplemented with 10% fetal calf serum, 0.25% lactalbumin hydrolysate, and 50 μg/ml gentamicin.

Animals

Pigs were housed in American Association for Laboratory Animal Care-approved biological containment facilities. Pigs were determined to be PRV seronegative prior to the start of the experiment by latex agglutination assay (Viral Antigens, Inc.). All animal manipulations were performed according to protocols approved by the Institutional Animal Care and Use Committee.

Intranasal inoculation of swine

Groups of nine 4-day-old male pigs were inoculated intranasally with 1×10^5 plaque-forming units (PFU)/nostril of either LLT$\beta\Delta$2 or LLTβres. At the designated times postinfection (p.i.), pigs were euthanized with a lethal dose of sodium pentobarbital, and the following tissues were collected: nasal turbinate, TG, spinal cord, tonsil, retropharyngeal, parotid, and tracheobronchial lymph nodes, trachea, thymus, lung, liver, spleen, kidney, adrenal, and blood and three blocks of tissue from the brain (cranial, middle, and caudal). The three blocks of tissue were produced by making coronal slices through the intact brain. The cranial block extended from the cruciate sulcus dorsally to the olfactory peduncle ventrally. The middle block went through the gyri and sulci of the endomarginal, marginal, ectomarginal, and suprasylvian areas and the piriform lobe. This plane included the lateral and third ventricles, the thalamus, and portions of the lateral geniculate body (diencephalon). The caudal block extended through the cerebellar vermis, primary fissure, dorsal and ventral paraflocculus, flocculus (dorsal metencephalon), peduncles, base of the trigeminal and vestibulocochlear nerves, and trapezoid body (ventral metencephalon).

Virus isolation

Suspensions of each tissue were prepared in 4.5 ml medium F15 containing penicillin (25 U/ml), streptomycin (25 μg/ml), neomycin sulfate (25 μg/ml), bacitracin (0.25 U/ml), and gentamicin sulfate (50 μg/ml), for virus isolation using a modification of the method described by McFerran and Dow (1965). PRV plaque assays were performed as described previously (Cheung *et al.,* 1994).

Pathology

Sections were fixed in a 10% neutral-buffered zinc–Formalin (Anatech, Ltd.) were routinely processed, cut into 5- to 8-μm sections, and stained with hematoxylin and eosin. Turbinate sections were decalcified with S/P decalcifying solution (Baxter Scientific Products), as recommended by the manufacturer. Microscopic lesions in each tissue were scored using a subjective scoring system based on inflammatory infiltrates, in comparison to age-matched uninfected control pigs. Since lesions of cerebellar folia lacked infiltrates, but contained areas of degeneration, a separate scoring system was used.

Intracranial inoculation of swine

Two-day-old piglets (three per group) were inoculated intracranially into the left cerebral hemisphere with serial 10-fold dilution of virus (30 μl) using a 26-gauge needle. The injection location was approximately 3–4 mm lateral to the midline and 2–4 mm caudal to a line connecting the posterior points of the eye orbits. PRV virulence was assessed by monitoring clinical signs and mortality. Spread of PRV from CNS to oronasal epithelium was monitored by plaque assay titration of nasal and oropharyngeal swab samples as previously described (Cheung *et al.,* 1994).

Immunohistochemistry

Tissues were fixed in HistoChoice (Amresco) and embedded in paraffin. Pseudorabies virus antigen was detected using a method previously described, except that tissue sections were not subjected to hydrated autoclaving (Miller *et al.,* 1993, 1994). A rabbit antiserum raised against live PRV (gift of Dr. Gene Pirtle, Iowa State Univer-

sity, Ames, IA) was used as the primary antibody. Tissues were incubated with a 1:1000 dilution of primary antibody at 4°C overnight.

RESULTS

Intranasal inoculation experiment

Pigs were infected as described under Materials and Methods and monitored three times daily. Virus content, distribution of microscopic lesions, viral antigen, and viral genomic DNA were analyzed in tissues collected from pigs necropsied at several different times p.i. For comparison, tissues also were collected at each time point from three age-matched uninfected control pigs. Three pigs per group were necropsied on Days 2 and 5 p.i. The remaining three LLTβres-infected pigs were necropsied on Day 7 p.i., because they were moribund. To determine if the LLT$\beta\Delta$2 would succumb to infection or recover, the remaining three LLT$\beta\Delta$2-infected and control uninfected pigs were necropsied on Day 9 p.i., at which point they were recovering.

Clinical signs

All uninfected control pigs remained healthy throughout the experiment. LLTβres-infected pigs developed clinical signs typical of PRV infection, beginning with anorexia and respiratory signs (nasal discharge, sneezing), starting at 3 days p.i., and progressing to severe respiratory signs and neurological signs (tremors, ataxia, paralysis). On Day 7 p.i., of the three remaining LLTβres-infected pigs, one was dead (No. 27) and the other two (Nos. 28 and 29) were euthanized, because they were moribund with severe respiratory and neurological signs. LLT$\beta\Delta$2-infected pigs were anorexic 3–4 days p.i. and developed respiratory signs on Day 5 p.i. One LLT$\beta\Delta$2-infected pig (No. 14) exhibited transitory vomiting starting less than 18 hr p.i. As it appeared so soon after infection, the vomiting observed was not characteristic of, and was not likely directly attributable to, PRV infection. By Day 7 p.i., all of the LLT$\beta\Delta$2-infected pigs were recovering and eating well. The remaining three LLT$\beta\Delta$2-infected pigs appeared clinically normal when they were necropsied on Day 9 p.i. Because the last necropsy day was different for the LLT$\beta\Delta$2 and LLTβres groups, no direct comparisons could be made between data obtained on Days 7 and 9 p.i.

Virus content of tissues from LLT$\boldsymbol{\beta\Delta}$2- and LLT$\boldsymbol{\beta}$res-infected pigs

All tissues collected from LLT$\beta\Delta$2- and LLTβres-infected pigs were processed for virus isolation. Virus isolated from LLT$\beta\Delta$2-infected pigs formed blue plaques in the presence of bluogal, indicating that LLT$\beta\Delta$2 was stable after one passage in pigs and that the virus isolated did not arise from contamination by other PRV strains. LLT$\beta\Delta$2 and LLTβres were isolated primarily from turbinate, tonsil, TG, CNS, lymph nodes, and lung (Table 1). Both viruses were isolated only sporadically from adrenal, spleen, thymus, and trachea (not shown), while neither virus was isolated from blood, liver, or kidney.

Virus content of tissues at the site of inoculation was comparable for LLT$\beta\Delta$2- and LLTβres-infected pigs. High titers of virus were isolated from nasal turbinate and tonsil from both LLT$\beta\Delta$2- and LLTβres-infected pigs. Interestingly, virus was isolated from lung earlier and more frequently in LLT$\beta\Delta$2-infected pigs than in LLTβres-infected pigs.

In TG, virus content of LLT$\beta\Delta$2-infected tissue was comparable to or greater than that in LLTβres-infected tissue. On Day 2 p.i., high titers of LLT$\beta\Delta$2 were present in TG of all three pigs, compared to only one LLTβres-infected pig. On Day 5 p.i., the titers of virus isolated from TG of LLT$\beta\Delta$2- and LLTβres-infected pigs were comparable.

LLT$\beta\Delta$2 was isolated from brain, but at lower titers than in LLTβres-infected brain. Significantly, virus was isolated from CNS of all three LLT$\beta\Delta$2-infected pigs on Day 5 p.i. Virus was isolated from the cranial and middle brain sections (olfactory bulb, frontal lobe, cerebrum) of three of three animals, the caudal brain section (cerebellum and brainstem) of two of three animals, and the spinal cord of one of three animals. Compared to LLTβres-infected tissues, the levels of virus were lower in LLT$\beta\Delta$2-infected brain sections. On average, PFU of virus isolated from LLT$\beta\Delta$2-infected brain was reduced 18-fold for the cranial section, 21-fold for the middle section, and 141-fold for the caudal section.

On Day 7 p.i., high titers of virus were present in the tissues shown in Table 1 from all LLTβres-infected pigs and these pigs were either moribund or dead. In contrast, by Day 9 p.i., virus was consistently isolated only from tonsils of LLT$\beta\Delta$2-infected pigs. The level of LLT$\beta\Delta$2 in the CNS declined between days 5 and 9 p.i., and the LLT$\beta\Delta$2-infected pigs never displayed clinical signs indicative of CNS infection.

Pathology produced by LLT$\boldsymbol{\Delta}$2 and LLT$\boldsymbol{\beta}$res

Lesions were observed in turbinate, TG, and CNS (Table 2) and were similar to previous reports of experimental PRV infection (McFerran and Dow, 1965). In tissues at the site of inoculation, the type and distribution of lesions were similar between LLT$\beta\Delta$2- and LLTβres-infected pigs, but, in general, the lesions produced by LLTβres were more severe. Crypt inflammation was observed in tonsils of all pigs, including control uninfected pigs, and was interpreted as incidental. Lymphocyte necrosis was also observed in tonsil of two LLTβres-infected pigs (27 and 29), but not in any LLT$\beta\Delta$2-infected pigs. In turbinate sections, exudate and necrosis was observed sooner and in greater severity in LLTβres-infected pigs than in LLT$\beta\Delta$2-infected pigs. Therefore, al-

TABLE 1

Virus Isolation from Tissues of PRV-Infected Pigs

		Log PFU/gram tissue										
									Brain			
	Pig No.	Turbinate	Tonsil	RLN[a]	PLN[b]	TBLN[c]	Lung	TG	Cranial	Middle	Caudal	Spinal cord
LLT$\beta\Delta$2												
	11	3.2	2.4	0	0	0	2.3	4.9	0	0	0	0
Day 2	12	3.6	3.0	0	0	0	0	3.6	0	0	0	0
	13	2.5	2.8	3.3	0	0	0	3.0	0	0	0	0
	14	>4.7	>6.7	5.0	3.4	0	4.0	5.4	3.4	2.2	2.6	2.2
Day 5	15	4.0	6.5	5.2	>5.7	0	2.8	4.6	2.9	2.4	3.3	0
	16	4.7	6.1	4.5	4.6	0	4.0	4.5	2.2	3.3	0	0
	17	0	4.7	0	0	0	0	0	0	0	0	0
Day 9	18	0	4.7	0	0	0	0	0	0	0	0	
	19	0	4.0	0	3.2	0	3.8	4.4	0	0	0	0
LLTβres												
	21	2.2	2.2	0	0	0	0	0	0	0	0	
Day 2	22	4.6	2.3	5.7	4.4	0	0	5.4	0	0	0	0
	23	0	2.9	0	0	0	0	0	0	0	2.8	2.0
	24	3.5	6.5	>5.7	>5.7	4.6	0	4.5	4.0	3.6	4.7	0
Day 5	25	4.2	>6.7	3.7	>5.7	>5.7	3.1	6.2	4.1	3.7	>4.7	2.5
	26	4.1	6.3	3.2	>5.7	0	0	6.2	2.5	3.0	3.5	2.0
	27	>4.7	>4.7	>5.7	>5.7	5.4	4.4	5.3	4.4	4.3	>4.7	4.2
Day 7	28	3.3	>4.7	>5.7	>5.7	5.3	2.8	4.2	3.2	3.1	4.4	0
	29	>4.7	>4.7	4.1	4.8	4.8	4.1	4.3	3.2	>4.7	4.3	2.7

[a] Retropharyngeal lymph node.
[b] Parotid lymph node.
[c] Tracheobronchial lymph node.

though pathology was noted in both groups, in turbinate and tonsils, lesions in the LLTβres-infected group were generally more severe (necrotizing) than those in the LLT$\beta\Delta$2 group.

In TG, the type, distribution, and severity of lesions were similar between LLT$\beta\Delta$2- and LLTβres-infected pigs. Lesions in TG were minimal on Day 2 p.i. Lesions were observed in TG of five of six LLT$\beta\Delta$2-infected animals on Days 5 and 9 p.i. and six of six LLTβres-infected animals on Days 5 and 7 p.i. These lesions consisted predominantly of moderate multifocal infiltrates of lymphocytes, macrophages, and occasional plasma cells in the perineuronal stroma (Fig. 1A).

Both LLT$\beta\Delta$2 and LLTβres produced lesions in pons, frontal lobe, cerebrum, and spinal cord. Lesions in brain were minimal in both LLT$\beta\Delta$2- and LLTβres-infected pigs on Day 2 p.i. The number of pigs with lesions and the severity of lesions were similar between the two groups at 2 days p.i. Central nervous system lesions were observed in brain of four of six LLT$\beta\Delta$2-infected pigs on Days 5 and 9 p.i. and six of six LLTβres-infected pigs on Days 5 and 7 p.i. Lesions in the spinal cord were observed less frequently in pigs from both groups. At the later time points, the distribution of lesions was similar in LLT$\beta\Delta$2- and LLTβres-infected pigs; however, brain lesions were generally more severe in pigs infected with LLTβres. Cerebral lesions were present in both gray and white matter of the cranial section and largely consisted of perivascular infiltrates and multifocal aggregates of lymphocytes, reactive astrocytes, and macrophages (glial nodules) in the frontal lobe and olfactory peduncle (Fig. 1B). Lesions also were observed in the vicinity of pyramidal neurons of the cerebral cortex. Most perivascular infiltrates were present in areas adjacent to the lateral and third ventricles of the middle section of tissue, whereas small hypercellular lesions of lymphocytes, reactive astrocytes, macrophages, and occasional neutrophils were present in multifocal distribution in the gray matter. In the caudal brain section, mild mononuclear perivascular lesions were present in the neuroparenchyma of the pons.

No cerebellar lesions were observed in LLT$\beta\Delta$2-infected pigs. However, lesions were present in cerebellar folia of the LLTβres-infected pigs at 5 and 7 days p.i. These lesions were focal, up to 500 μm in length, and extended from the Purkinje layer to varying depths within the granular layer. Neurons often had deeply basophilic nuclei, rounded cell borders with eosinophilic cytoplasm, and were separated by modest amounts of edema. Mild perivascular lymphocytic infiltrates were present in the cerebellar folia of only one pig.

Tissue distribution of PRV antigen in LLT$\beta\Delta$2- and LLTβres-infected pigs

The localization of PRV antigen in infected tissues was determined by immunohistochemical examination. Antigen

TABLE 2

Histopathological Lesion Scores of Tissues from PRV-Infected Pigs

	Pig No.	Turbinate[a]	TG[b]	Frontal lobe	Middle cerebrum	Pons	Cerebellum[c]	Spinal cord
LLT$\beta\Delta$2								
	11	E	0	0	1	0	−	0
Day 2	12	E	0	0	0	0	−	0
	13	E	0	0	1	0	−	0
	14	E	0	0	0	0	−	0
Day 5	15	E	1	1	2	2	−	0
	16	R	3	1	2	3	−	0
	17	E	3	0	0	0	−	0
Day 9	18	0	2	2	3	0	−	0
	19	E	2	3	3	2	−	1
LLTβres								
	21	0	2	0	0	0	−	0
Day 2	22	0	0	0	0	0	−	0
	23	E	0	0	3	0	−	0
	24	R	3	3	3	2	+	0
Day 5	25	R	1	0	2	2	−	0
	26	R	2	3	3	2	+	0
	27	R	3	3	3	2	++	0
Day 7	28	R	3	1	0	2	+	2
	29	R	2	3	4	3	++	3

[a] E, exudate, where no mucosal damage was present but exudate was present in the nasal meatus; R, rhinitis, where inflammation and/or necrosis of the nasal mucosa was observed.

[b] Microscopic lesions in the sections of brain, TG, and spinal cord were classified using a predetermined subjective scoring system. Scores varied on a 0–5 scale: no remarkable lesions (0), minimal (1), mild (2), moderate (3), marked (4), and severe (5). Criteria for each category were: minimal, an influx of a single layer or less of mononuclear cells into perivascular spaces of one or more vessels of the parenchyma or meninges; mild, layer one to three cells thick of mononuclear cells into the perivascular areas and formation of reactive astrocytes with neuronal satellitosis; moderate, dense infiltrate of inflammatory cells into perivascular spaces with extension into the adjacent parenchyma and the formation of nodular inflammatory lesions with mononuclear cells, reactive astrocytes, satellitosis, and neuronal necrosis; marked, dense inflammatory infiltrates and reactive astrocytes throughout the section with small areas of parenchymal necrosis; and severe, dense inflammatory infiltrates with extensive areas of parenchymal necrosis.

[c] Lesions of the cerebellar folia were scored with a separate scale: no lesion (−), mild nuclear basophilia and edema (+), and one focal area of necrosis $<$300 μm wide (++).

was detected at the site of inoculation (turbinate and tonsil) and in draining lymph nodes, TG, and CNS (Table 3). The results of immunohistochemical analysis were verified by *in situ* hybridization using PRV genomic DNA as a probe (not shown). Antigen was not detected in other tissues, with the exception of single, small foci of staining in thymus of pig 19 and lungs of pigs 19, 25, and 27.

The distribution and quantity of antigen in turbinate and tonsil was comparable between LLT$\beta\Delta$2- and LLTβres-infected pigs. On Day 2 p.i., antigen was detected only in very few turbinate and tonsil sections. By Day 5 p.i., antigen was present in turbinates and tonsils of all LLT$\beta\Delta$2- and LLTβres-infected pigs. A typical tonsil section had multiple foci of stained mucosal epithelial cells, several of which also involved underlying lymphoid tissue (Fig. 2A). In turbinate sections, staining was typically observed in multiple foci of epithelial cells or adjacent to osseous and cartilaginous trabeculae (Fig. 2B). In turbinate sections, antigen was detected less often in tooth pulp, nerves, and hard palate epithelium from both LLT$\beta\Delta$2- and LLTβres-infected pigs. Antigen was present in turbinates and tonsils of all LLTβres-infected pigs on Day 7 p.i., but only the turbinate of one LLT$\beta\Delta$2-infected pig was positive on Day 9 p.i.

Antigen was first detected in TG on Day 5 p.i., and the amount of antigen detected was comparable between LLT$\beta\Delta$2- and LLTβres-infected pigs. Reactivity was localized primarily in neurons (Figs. 2C and 2D). On Day 7 p.i., only a few positive neurons were detected in TG of LLTβres-infected pigs, and none were detected in the LLT$\beta\Delta$2-infected pigs on Day 9 p.i.

While widespread antigen was present in the brain of LLTβres-infected pigs, very few antigen-positive cells were detected in cerebrum of LLT$\beta\Delta$2-infected pigs, and antigen was not detected in cerebellum. Antigen was first detected in brain sections on Day 5 p.i. Brain tissues from pigs infected with LLTβres contained moderate to large amounts and widespread distribution of antigen. The cells most consistently stained were pyramidal cell neurons of the cerebral cortex (Fig. 3A). Antigen-positive neurons and other cells also were observed in the cerebrum, sometimes occurring in groups, but more often as single isolated cells. This staining pattern was characteristic for both the cranial and middle sections of the cere-

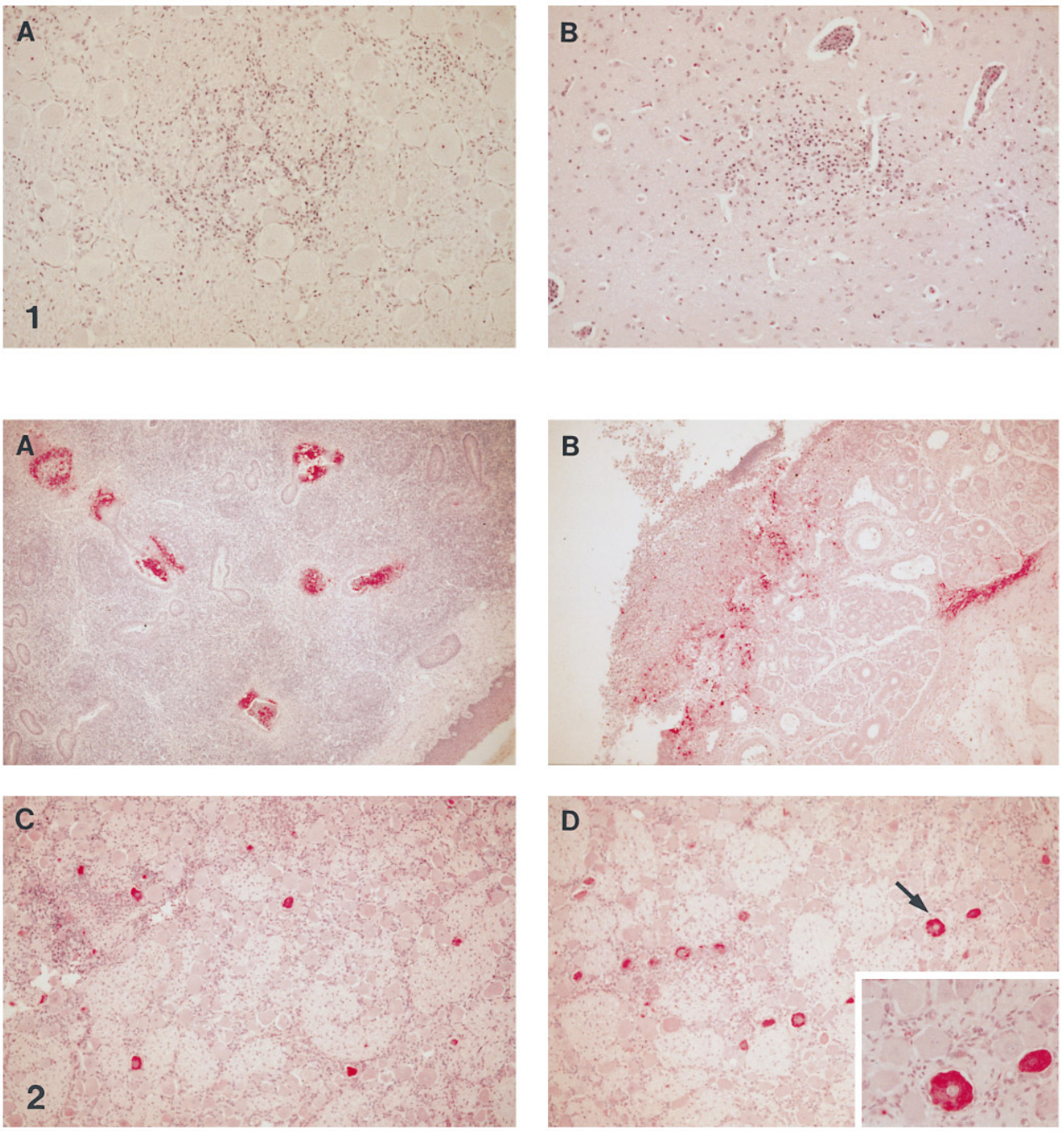

FIG. 1. Brain and trigeminal ganglion lesions in LLT$\beta\Delta$2-infected pigs. The lesions shown were typical of what was observed in two of three LLT$\beta\Delta$2-infected pigs at 5 days p.i. Lesions observed in LLTβres-infected pigs at 5 days p.i. were more severe (Table 2). (A) Focal inflammatory response in the TG of pig 15. (B) Glial nodule and perivascular cuffing in the frontal lobe of pig 14.

FIG. 2. Immunohistochemical staining (red) of PRV antigen in tonsil, turbinate and trigeminal ganglion at 5 days p.i. The distribution and amount of antigen detected in each tissue was comparable among LLT$\beta\Delta$2- and LLTβres-infected pigs. (A) Tonsil section from LLT$\beta\Delta$2-infected pig 15 demonstrating antigen in tonsillar crypts and underlying lymphoid tissue. (B) Turbinate section from LLT$\beta\Delta$2-infected pig 15 showing antigen in mucosal epithelial cells and underlying tissue. (C and D) Trigeminal ganglion sections from LLT$\beta\Delta$2-infected pig 16 (C) and LLTβres-infected pig 25 (D), demonstrating antigen localization in neurons and inflammatory response in the vicinity of infected neurons.

brum of all LLTβres-infected pigs. In caudal brain sections, staining was consistently observed in the cerebellum, either in isolated Purkinje cells or in foci that centered on the Purkinje cell layer and frequently extended into the granular and molecular cell layers (Fig. 3C). Staining was observed less frequently in white matter. Only rare positive cells were seen in the area of brain stem beneath the cerebellar peduncle.

Although antigen was detected in brain of all LLT$\beta\Delta$2-infected pigs on Day 5 p.i., the amount and distribution differed markedly from what was observed for the LLTβres-infected group. Generally, very few positive

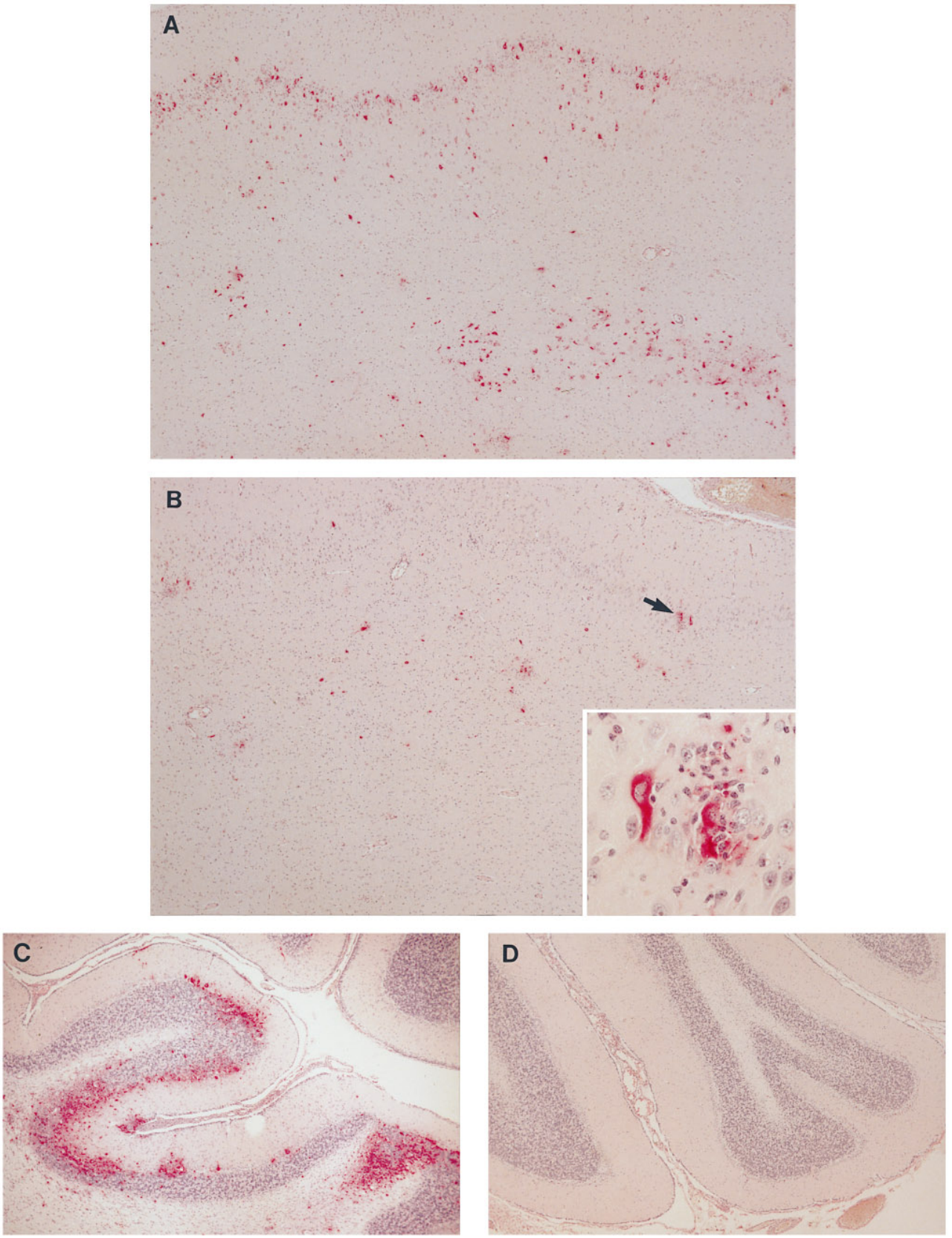

FIG. 3. Immunohistochemical staining of PRV antigen in brain tissues of LLT$\beta\Delta$2- and LLTβres-infected pigs at 5 days p.i. (A) Typical distribution of antigen in pyramidal neurons of the cerebral cortex and adjacent nuclei in LLTβres-infected pig 25. (B) Distribution of antigen in the vicinity of pyramidal neurons of the cerebral cortex in LLT$\beta\Delta$2-infected pig 16. This section shows the maximal staining observed in brain tissue from all LLT$\beta\Delta$2-infected pigs. Only a few antigen-positive cells were observed in brain sections from the other two LLT$\beta\Delta$2-infected pigs necropsied at 5 days p.i. (C) Typical distribution of antigen in Purkinje cells, and granular and molecular cell layers of the cerebellum in LLTβres-infected pig 25. (D) Absence of antigen in cerebellum of LLT$\beta\Delta$2-infected pig 15. No antigen was detected in cerebellum of any other LLT$\beta\Delta$2-infected pig.

TABLE 3

Immunohistochemical Detection of PRV Antigen from Tissues of PRV-Infected Pigs[a]

	Pig No.	Turbinate	Tonsil	RLN[c]	PLN[d]	TG	Frontal lobe	Middle cerebrum	Cerebellum
LLT$\beta\Delta$2									
	11	–[b]	–	–	–	–	–	–	–
Day 2	12	1	–	–	–	–	–	–	–
	13	–	–	–	–	ND[e]	–	–	–
	14	2	2	2	1	2	–	1	–
Day 5	15	2	2	–	1	1	–	1	–
	16	2	1	–	–	2	2	1	–
	17	–	1	–	–	–	–	–	–
Day 9	18	–	–	–	–	–	–	–	–
	19	2	–	–	–	–	1	1	–
LLTβres									
	21	–	–	–	–	–	–	–	–
Day 2	22	–	–	1	1	–	–	–	–
	23	–	1	–	–	–	–	–	–
	24	2	3	2	1	1	4	4	2
Day 5	25	1	1	–	–	2	2	2	2
	26	3	2	–	1	2	2	2	2
	27	3	3	1	1	2	4	4	2
Day 7	28	1	3	1	1	1	4	4	1
	29	1	1	1	–	1	3	4	1

[a] Positive tissues not shown on the table, TBLN of pig 27, 1; TBLN of pig 28, 2. Tissues from uninfected control pigs were all negative.

[b] (–) No staining; (1) a few to several areas of focal staining, most foci involving fewer than 5 cells (frequently only 1 or 2); (2) a few to several foci of staining, most foci involving 5–20 cells; (3) many foci of 5–20 stained cells or a few foci of >20 stained cells; (4) many foci, each with >20 cells scattered throughout a large area of the tissue.

[c] Retropharyngeal lymph node.

[d] Parotid lymph node.

[e] ND, not done.

cells were observed in brain tissues of pigs infected with LLT$\beta\Delta$2. Some of the stained cells appeared to be neurons. These cells were observed most commonly in cerebrum, in the vicinity of the pyramidal cell neurons (Fig. 3B). The cerebral section shown in Fig. 3B contained the highest concentration of antigen-positive cells observed in brain tissue for any LLT$\beta\Delta$2-infected pig. Antigen was not detected in the cerebellum of LLT$\beta\Delta$2-infected pigs (Fig. 3D).

Intracranial inoculation experiment

The intranasal inoculation experiment suggested that either LLT$\beta\Delta$2 was reduced in spread from TG to the CNS or was reduced in ability to replicate in CNS tissues. To determine if LLT$\beta\Delta$2 could replicate in CNS tissues, 2-day-old pigs were inoculated intracranially with doses of LLT$\beta\Delta$2 or LLTβres. At the two higher doses, no differences were noted in clinical signs or time to death (Table 4). At the two lower doses (2×10^2 and 2×10^3 PFU), neurological signs and disease progression occurred more quickly in LLTβres-infected pigs than in LLT$\beta\Delta$2-infected pigs. All LLTβres-infected pigs were dead or moribund and therefore euthanized by 84 hr p.i., whereas 4 of 6 LLT$\beta\Delta$2-infected pigs lived beyond 96 hr p.i., including 1 pig (15) which remained healthy through 132 hr p.i. On Day 2 p.i., virus was isolated from nasal and oropharyngeal swab samples of 9/11 LLTβres-infected pigs and 5/7 LLT$\beta\Delta$2-infected pigs.

Immunohistochemical examinations of cerebrum were performed on 1 pig in each of the groups inoculated intracerebrally with 10-fold dilutions of LLTβres (Table 4) (pigs 3, 5, 7, and 10) or LLT$\beta\Delta$2 (pigs 13, 18, 21, and 24). All pigs inoculated with LLTβres and pig 21, which received LLT$\beta\Delta$2, had large amounts of antigen. Less antigen was observed in pigs 13 and 18 and antigen was not detected in pig 24, which was inoculated with the highest dose of LLT$\beta\Delta$2.

DISCUSSION

After intranasal infection, LLT$\beta\Delta$2 replicated and spread efficiently in tissues at the site of inoculation. The virus established infection in nasal and tonsillar epithelial cells and spread to underlying tissues at both sites. Notably, LLT$\beta\Delta$2 spread to and replicated to high titer in TG. LLT$\beta\Delta$2 also spread to a limited extent to CNS, as demonstrated by isolation of virus and observation of microscopic lesions in LLT$\beta\Delta$2-infected brain, in the vicinity of up to third order neurons (pyramidal neurons of the cerebral cortex). However, the widespread distribu-

TABLE 4

Intracranial Inoculation of Neonatal Pigs

		A. Time of death Postinfection (hr)								B. Virus recovery at oronasal pharyngeal epithelium Postinfection (day)			
	Pig No.	24	36	48	60	72	84	96	108	1	2	3	4
LLTβres (PFU)													
2.1×10^2	1					E				−	+++		
	2						D			−	+++	+++	
	3						E			−	+++	+++	
2.1×10^3	4						E			−	−	+++	
	5				E					−	+++		
	6					E				−	+++		
2.1×10^4	7					E				−	+++		
	8					E				−	+++		
	9					E				−	+++		
2.1×10^5	10					E				−	−		
	11				D					−	+++		
	12				D					−	ND		
LLT$\beta\Delta$ (PFU)													
2.1×10^2	13						D			−	+++	+++	
	14								E	−	+	+	+++
	15								H	−	−	−	++
2.1×10^3	16							D		−	+	+	++
	17								E	−	+	++	+++
	18[a]				D					−	−		
2.1×10^4	(19)									−	N/A		
	20[a]			D						−	ND		
	21					E				−	ND		
2.1×10^5	22				D					−	ND		
	23			D						−	ND		
	24					E				−	+		

Note. [a]1-day-old pig (younger, more susceptible). () Pig died of trauma, unrelated to PRV. N/A Not applicable. E, euthanized; D, death; H, healthy; ND, not done; (−) no virus; (+) 1–30 PFU; (++) 30–200 PFU; (+++) >200 PFU.

tion of PRV antigen observed in neurons and other cells, and high virus titers in LLTβres-infected brain tissues were not seen in LLT$\beta\Delta$2-infected-brain tissues.

In contrast, when inoculated directly into the cerebral cortex of swine, LLT$\beta\Delta$2 was virulent. Compared to LLTβres-infected pigs, LLT$\beta\Delta$2-infected pigs exhibited delayed neurological signs and death and a reduction in viral antigen content in the brain; however, all but one LLT$\beta\Delta$2-infected pig died. These results demonstrate that the reduced virulence of LLT$\beta\Delta$2 observed after intranasal inoculation is not due to inability to replicate in CNS tissues. Rather, because LLT$\beta\Delta$2 replicated efficiently in TG the defect may be a reduced ability to spread from TG neurons (and possibly other peripheral neurons) to CNS neurons. Mulder *et al.* (1994) reported a similar pattern of restricted spread in studies of a gE-negative PRV and concluded that inhibition resulted from a deficient infection of second- and third-order CNS neurons. Interestingly, after intracranial inoculation, LLT$\beta\Delta$2 spread from CNS to peripheral tissues, as evidenced by nasal and oropharyngeal virus shedding. This observation indicates that spread from second- and third-order CNS neurons to first-order neurons was not inhibited.

While wild-type PRV strains (Mulder *et al.,* 1994; this study) and LLTβres spread from TG to the cerebellum after intranasal inoculation, neither antigen nor lesions were present in the cerebellum of LLT$\beta\Delta$2-infected pigs after intranasal inoculation. Absence of LLT$\beta\Delta$2 antigen in the cerebellum after intranasal inoculation was most likely due to the limited amount of virus in cortical neurons available to spread to the cerebellum.

Analysis of recombinant strains of HSV and PRV has identified several types of defects which result in reduced neuroinvasiveness or neurovirulence. For both viruses, altered expression of some viral glycoproteins reduces neuroinvasiveness (Babic *et al.,* 1993; Card *et al.,* 1992; Kimman *et al.,* 1992; Mulder *et al.,* 1994; Stevens, 1991). However, the glycoproteins involved differ between HSV and PRV. For example, a single amino acid mutation in glycoprotein D (gD) of HSV significantly alters neuroinvasiveness (Izumi, 1990), while deletion of PRV gD has no effect on spread of PRV to and within the CNS in mice (Babic, 1993). Replication of another class of mutants reduced in neurovirulence or neuroinvasiveness is dependent on the physiological state of the cell. Mutations in HSV genes, such as thymidine kinase, dUTPase,

or ICP34.5, are thought to restrict virus replication only in nondividing cells, such as neurons, presumably because their functions are complemented by cellular factors in dividing cells (Stevens, 1991). Other phenotypes of mutants which are reduced in neurovirulence have also been described. For example, a recombinant strain of HSV-1 which expresses the HSV-2 form of UL5 helicase protein is restricted for replication in neuronal cells of mice, yet replicates to high titers in nonneuronal cells in CNS and sensory ganglia (Bloom and Stevens, 1994; Javier *et al.,* 1988).

The altered neurovirulence/neuroinvasiveness of LLT$\beta\Delta$2 maps to the PRV UL/IR junction. Known genes disrupted by the deletion include LLT and IE180. In addition, there are several complex and incompletely characterized transcripts overlapping or in the vicinity of the deletion (Cheung, 1990). Further studies will be required to determine to which gene the altered neurovirulence/neuroinvasiveness of LLT$\beta\Delta$2 can be ascribed. Genes in the vicinity of the UL/IR junction have been shown to modulate neurovirulence in other herpesviruses. The HSV ICP34.5 gene has been associated with neurovirulence and is in a comparable genomic location. However, no open reading frame with detectable homology to ICP34.5 could be identified in the PRV sequences. In addition, the phenotype of LLT$\beta\Delta$2 in swine differs from the phenotype of ICP34.5 mutants in mice. Replication of ICP34.5 mutants is reduced in CNS, peripheral neurons, and other tissues (Bolovan *et al.,* 1994; Chou *et al.,* 1990), while the restriction in LLT$\beta\Delta$2 is specific for CNS and not TG.

In summary, this study demonstrated that LLT$\beta\Delta$2 is neurovirulent, since it replicated and caused death after intracranial inoculation. However, LLT$\beta\Delta$2 is reduced in virulence after intranasal inoculation, despite replication to high titer in TG neurons. Further investigation will be required to assign the phenotype of LLT$\beta\Delta$2 to a specific gene.

ACKNOWLEDGMENTS

The authors thank Dr. Gene Pirtle for the anti-PRV reagent, Dennis Orcutt for technical assistance, and Gene Hedberg, Wayne Romp, and Tom Glasson for photography.

REFERENCES

Babic, N., Mettenleiter, T. C., Flamand, A., and Ugolini, G. (1993). Role of essential glycoproteins gII and gp50 in transneuronal transfer of pseudorabies virus from the hypoglossal nerves of mice. *J. Virol.* 67, 4421–4426.

Babic, N., Mettenleiter, T. C., Ugolini, G., Flamand, A., and Coulon, P. (1994). Propagation of pseudorabies virus in the nervous system of the mouse after intranasal inoculation. *Virology* 204, 616–625.

Bloom, D., and Stevens, J. G. (1994). Neuron-specific restriction of a herpes simplex virus recombinant maps to the UL5 gene. *J. Virol.* 68, 3761–3772.

Bolovan, C. A., Sawtell, N. M., and Thompson, R. L. (1994). ICP34.5 mutants of herpes simplex virus type 1 strain 17syn+ are attenuated for neurovirulence in mice and for replication in confluent primary mouse embryo cell cultures. *J. Virol.* 68, 48–55.

Card, J. P., and Enquist, L. W. (1994). Use of pseudorabies virus for definition of synaptically linked populations of neurons. *In* "Methods in Molecular Genetics, Vol. 4: Molecular Virology Techniques, Part A" (W. Adolph, Ed.), pp. 363–382. Academic Press, New York.

Card, J. P., Rinaman, L., Schwaber, J. S., Miselis, R. R., Whealy, M. E., Robbins, A. K., and Enquist, L. W. (1990). Neurotropic properties of pseudorabies virus: Uptake and transneuronal passage in the rat central nervous system. *J. Neurosci.* 10(66), 1974–1994.

Card, J. P., Whealy, M. E., Robbins, A. K., and Enquist, L. W. (1992). Pseudorabies virus envelope glycoprotein gI influences both neurotropism and virulence during infection of the rat visual system. *J. Virol.* 66, 3032–3041.

Cheung, A. K. (1990). The *Bam*HI J fragment (0.706 to 0.737 map units) of pseudorabies virus is transcriptionally active during viral replication. *J. Virol.* 64, 977–983.

Cheung, A. K., Fang, J., and Wesley, R. D. (1994). Characterization of a pseudorabies virus defective in the early protein 0 and latency genes. *Am. J. Vet. Res.* 55, 1710–1716.

Chou, J., Kern, E. R., Whitley, R. J., and Roizman, B. (1990). Mapping of herpes simplex virus-1 neurovirulence to gamma 34.5, a gene nonessential for growth in culture. *Science* 250, 1262–1266.

Dean, H. J., and Cheung, A. K. (1995). A deletion at the UL/IR junction reduces pseudorabies virus neurovirulence. *J. Virol.* 69, 1334–1338.

Izumi, K. M., and Stevens, J. G. (1990). Molecular and biological characterization of a herpes simplex virus type 1 (HSV-1) neuroinvasiveness gene. *J. Exp. Med.* 172, 487–496.

Javier, R. T., Izumi, K. M., and Stevens, J. G. (1988). Localization of a herpes simplex virus neurovirulence gene dissociated from high-titer virus replication in the brain. *J. Virol.* 62, 1381–1387.12.

Kimman, T. G., DeWind, N., Oei-Lie, N., Pol, J. M. A., Berns, A. J. M., and Gielkens, A. L. J. (1992). Contribution of single genes within the unique short region of Aujeszky's disease virus (suid herpesvirus type 1) to virulence, pathogenesis and immunogenicity. *J. Gen. Virol.* 73, 243–251.

Martin, X., and Dolivo, M. (1983). Neuronal and transneuronal tracing in the trigeminal system of the rat using the herpesvirus suis. *Brain Res.* 273, 253–276.

McFerran, J. B., and Dow, C. (1965). The distribution of the virus of Aujeszky's disease (pseudorabies virus) in experimentally infected swine. *Am. J. Vet. Res.* 26, 631–635.

Miller, J. M., Jenny, A. L., Taylor, W. D., Marsh, R. F., Rubenstein, R., and Race, R. E. (1993). Immunohistochemical detection of prion protein in sheep with scrapie. *J. Vet. Diagn. Invest.* 5, 309–316.

Miller, J. M., Jenny, A. L., Taylor, W. D., Race, R. E., Ernst, D. R., Katz, J. B., and Rubenstein, R. (1994). Detection of prion protein in formalin-fixed brain by hydrated autoclaving immunohistochemistry for the diagnosis of scrapie in sheep. *J. Vet. Diagn. Invest.* 6, 366–368.

Mulder, W. A. M., Jacobs, L., Priem, J., Kok, G. L., Wagenaar, F., Kimman, T. G., and Pol, J. M. A. (1994). Glycoprotein gE-negative pseudorabies virus has a reduced capability to infect second- and third-order neurons of the olfactory and trigeminal routes in the porcine central nervous system. *J. Gen. Virol.* 75, 3095–3106.

Rinaman, L., Card, J. P., and Enquist, L. W. (1993). Spatiotemporal responses of astrocytes, ramified microglia, and brain macrophages to central neuronal infection with pseudorabies virus. *J. Neurosci.* 13(2), 685–702.

Stevens, J. G. (1991). Herpes simplex virus: neuroinvasiveness, neurovirulence and latency. *Semin. Neurosci.* 3, 141–147.

Wittman, G., and Rziha, H.-J. (1989). Aujeszky's Disease (pseudorabies) in pigs. *In* "Herpesvirus Diseases of Cattle, Horses and Pigs" (G. Wittman, Ed.), pp. 230–325. Kluwer, Boston.